REMARKS

Entry of this amendment and reconsideration of the rejection of the claims is respectfully requested.

Claims 77-78, 121-130, and 132 have been cancelled without prejudice or disclaimer. Claims 121-130 are cancelled due to a restriction requirement and applicants reserve the right to pursue the subject matter of these claims in a divisional application. Applicants reserve the right to pursue the subject matter of claims 77-78 and 132 in one or more continuation applications

Claims 55, 70-71, 89, and 105 are currently amended to clarify the subject matter of the claims. These amendments are supported throughout the specification. No new matter is introduced by these amendments.

Interview Summary

Applicants thank Examiner Crowder and her supervisor for the interview on October 9, 2007. We discussed the art rejections of record.

Restriction

The Examiner indicated that claims 86, 100, and 121-130 have been withdrawn from consideration as being drawn to nonelected inventions.

While not acquiescing to the statements by the Examiner and solely to expedite prosecution, Applicants have cancelled claims 121-130 without prejudice or disclaimer.

With regard to claims 86 and 100, Applicants submit that these claims were withdrawn from examination due to species election requirement. Applicants request rejoinder of the claims and examination if the elected species is found allowable.

Objection to the Claims

Claim 132 was objected to for being dependent on non elected claims. While not acquiescing to the rejection and solely to expedite prosecution, claim 132 has been cancelled rendering the rejection moot.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 55, 58-68, 70-85, 87–99, 101, 103-105, 107-113,131 and 132 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the written description requirement. Claims 77-78, and 132 have been cancelled rendering the rejection of these claims moot. Applicants respectfully traverse the rejection with regard to claims 55, 58-68, 70-76, 79, 83-85, 87-99, 101, 103-105, 107-113 and 131.

Specifically, the Examiner contends that the specification fails to support the phrase "cysteine residue forms an inter-chain disulfide chain when present". While not acquiescing to the rejection and solely to expedite prosecution, the claims no longer refer to "forms". Applicants request withdrawal of the rejection

Rejections under 35 U.S.C. § 102(b)

Claims 55, and 58 were rejected under 35 U.S.C. § 102(b) as anticipated by Gillies et al., *Human Antibody Hybridomas* 1: 47–54 (1990), and by Davis et al., *EMBO J.* 9: 2519–2526 (1989). Applicants respectfully traverse this rejection.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Because neither Gillies nor Davis teach each and every element of the claims, they do not anticipate the claimed subject matter.

Gillies, inter alia, does not teach an isolated polynucleotide comprising a prokaryotic secretion signal sequence. Gillies et al teaches a construct expressed in a hybridoma cell line. Therefore, as Gillies fails to teach each and every element of the present claims, the reference does not anticipate the present claims.

Davies et al, inter alia, does not teach an isolated polynucleotide comprising a prokaryotic secretion signal sequence. Davies et al teaches a construct expressed in a hybridoma cell lines. Therefore, as Davis fails to teach each and every element of the present claims, the reference does not anticipate the present claims.

Based on the foregoing, withdrawal of the rejections under 35 U.S.C. § 102(b) is respectfully requested.

Rejection under 35 U.S.C. § 102(e)

Claims 55, 58–68, 70-85, 87–99, 101,103-105, 107-113 and 131-32 were rejected under 35 U.S.C. § 102(b) as anticipated by Simmons et al. (U.S. Patent Pub. No. 2005/0170464). Applicants respectfully traverse this rejection.

The present claims are directed to an isolated polynucleotide comprising a polynucleotide encoding a prokaryotic secretion signal sequence and a polynucleotide encoding an intact antibody comprising a variant heavy chain wherein the variant heavy chain comprises a variant hinge region which does not form inter-heavy chain disulfide linkages, and wherein said variant hinge region lacks a cysteine residue, wherein the cysteine residue is capable of forming an inter-chain disulfide linkage when present.

Applicants submit that when the Simmons reference is read as a whole, it does not disclose every element of the claims. The Simmons reference is directed to methods for improved expression and production of biologically active antibodies by using separate cistrons under the control of separate promoters and including separate translation initiation regions. The reference indicates that a cysteine residue not involved in maintaining the proper conformation of the antibody can be substituted by serine residues. It is important to note the reference does not state that any cysteine residue can be altered but those that are not involved in maintaining proper conformation of the antibody.

The Simmons et al reference describes the structure of full length antibodies throughout the document including at paragraph [0044] by stating "A naturally occurring antibody comprises four polypeptide chains, two identical heavy (H) chains and two identical light (L) chains, inter-connected by disulfide bonds." (emphasis added) At paragraph [0049] the reference states "The capability of a full length antibody to exert one or more of its natural activities depends on several factors, including proper folding and assembly of the polypeptide chains. As used herein, the biologically active immunoglobulins generated by the disclosed methods are typically heterotetramers having two identical L chains and two identical H chains that are linked by multiple disulfide bonds and properly folded." (emphasis added) At

paragraph [0103], the Simmons et al reference further states "Sufficient disulfide bonds are particularly important for the formation and folding of full length, bivalent antibodies having two heavy chains and two light chains." (emphasis added) Applicants submit when the Simmons reference is read as a whole, cysteine residues that form disulfide bonds that interconnect two heavy and light chains are involved in maintaining the proper conformation of a full length antibody. Therefore, the Simmons et al reference does not teach that an intact antibody can or should be produced wherein the variant heavy chain comprises a variant hinge region which does not form inter-heavy chain disulfide linkages, and wherein said variant hinge region lacks a cysteine residue, wherein the cysteine residue is capable of forming an inter-chain disulfide linkage when present as claimed by Applicants.

Secondly, Applicants submit that the Simmons et al reference indicates that a cysteine residue not involved in maintaining the proper conformation of the antibody can be substituted, generally by serine residues, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. One of skill in the art reading the Simmons et al reference would understand that aberrant crosslinking can occur when cysteines in the hinge region form intradomain rather than the inter heavy chain domain cross links. Brennan et al, Science 229:81 (1985) In addition, it is known to those of skill in the art that formation of proper disulfide bonds increases oxidative stability. See Missiakas et al, Journal of Bacteriology 179:2465 (1997) Thus, the Simmons reference as a whole does not disclose a polynucleotide that encodes an intact antibody comprising a variant heavy chain comprising a variant hinge region which does not form inter-heavy chain disulfide linkages, and wherein said variant hinge region lacks a cysteine residue, wherein the cysteine residue is capable of forming an inter-chain disulfide linkage when present could provide for an intact antibody in high yield.

Withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §103(a)

Claims 55, 57–85, 87–99, 101 and 103–114 were rejected under 35 U.S.C. § 103(a) as unpatentable over Gillies et al. and Davis et al., in view of Georgiou et al. (U.S. Patent No. 5,264,365) and Kurokawa et al., *J. Biol. Chem.* 276:14393–14399 (2001). Claims 77-78, 80-82 and 132 have been cancelled rendering the rejection of these claims moot. Applicants

respectfully traverse the rejection with regard to claims 55, 58-68, 70-76, 79, 83-85, 87-99, 101, 103-105, 107-113 and 131.

To make a *prima facie* case of obviousness, "it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed." *Id.* The initial burden to make a *prima facie* case of obviousness is on the Examiner. *In re Bell*, 991 F.2d 781, 783 (Fed. Cir. 1993). Applicants submit that the Examiner does not make a *prima facie* case of obviousness, because all the limitations of the present claims are not taught by the combination of references cited in the Office Action and one of skill in the art would not have a reason to combine the references.

The present claims are directed to an isolated polynucleotide comprising a polynucleotide that encodes a prokaryotic secretion signal sequence and a polynucleotide encoding an intact antibody comprising a variant heavy chain, wherein the variant heavy chain comprises a variant hinge region which does not form inter-heavy chain disulfide linkages, and wherein the variant hinge region lacks a cysteine residue, wherein the cysteine residue is capable of forming an inter-heavy chain disulfide linkage when present.

The arguments and remarks provided above with respect to the Gillies and Davis references are also fully relevant here and are incorporated by reference to avoid repetition. To briefly summarize, Gillies fails to teach or suggest an isolated polynucleotide comprising a prokaryotic secretion signal sequence. Davis fails to teach or suggest an isolated polynucleotide comprising a prokaryotic secretion signal sequence. Georgiou is directed to construction of protease deficient *E. coli* hosts used to produce proteolytically sensitive peptides. Kurokawa describes production of NGF in *E. coli*, and the effect of Dsb protein overexpression on NGF production. None of these references, when combined, teach or suggest a polynucleotide comprising a polynucleotide encoding a prokaryotic secretion signal sequence and a polynucleotide encoding an intact antibody comprising a variant heavy chain, wherein the variant heavy chain comprises a variant hinge region that does not form inter-heavy chain disulfide linkages.

Moreover, one of skill in the art would not have a reason to combine these references. Gillies is focused on the interaction of effector functions and antigen binding of these constructs

produced in hybridoma cells. Gillies does not discuss whether these antibody constructs affect heavy chain aggregation or can increase antibody production. In fact, expression of the antibody constructs as described by Gillies et al shows formation of a heavy chain dimer due to aberrant crosslinking between the cysteines that normally are cross linked to the light chain. See figure 5. In addition, Gillies et al indicates that they did not form an intact antibody from the construct in which the cysteines were mutated. Thus, one of skill in the art interested in producing an intact antibody would not follow the teachings of Gillies because they did not produce any intact antibody. In addition, Gillies does not teach or suggest that intact antibodies can be produced in prokaryotic cells. As discussed in Missiakas et al, the production of disulfide containing proteins in bacteria is complex and susceptible to environmental factors.

The deficiency of Gillies is not remedied by reference to Georgiou, the '365 patent. The '365 patent does not describe production of intact antibodies nor does it describe or show a construct with a prokaryotic secretion signal sequence. The exemplified protein was a fusion of two bacterial proteins therefore it is unclear whether such secretion signal sequence was present or whether such secretion signal sequence would function to provide for secretion of intact antibodies as claimed by applicants.

The deficiency of Gillies is also not remedied by reference to Kurokawa et al. The Kurokawa et al reference is directed to expression of nerve growth factor and enhancement of disulfide bond formation. This reference does not teach or suggest a polynucleotide that encodes a prokaryotic secretion signal sequence and a polynucleotide encoding an intact antibody comprising a variant heavy chain, wherein the variant heavy chain comprises a variant hinge region which does not form inter-heavy chain disulfide linkages. The Kurokawa reference describes enhancing the disulfide bond formation.

Davis describes assembly of IgM subunits. Davis fails to teach or suggest an isolated polynucleotide comprising a prokaryotic secretion signal sequence. In addition, Davis et al. does not teach or suggest that intact antibodies can be produced in prokaryotic cells. As discussed in Missiakas et al., the production of disulfide containing proteins in bacteria is complex and susceptible to environmental factors.

The deficiency of Davis is not remedied by reference to Georgiou, the '365 patent. The '365 patent does not describe production of intact antibodies nor does it describe or show a construct with a prokaryotic secretion signal sequence. The exemplified protein was a fusion of two bacterial proteins therefore it is unclear whether such secretion signal sequence was present or whether such secretion signal sequence would function to provide for secretion of intact antibodies as claimed by applicants.

The deficiency of Davis is also not remedied by reference to Kurokawa et al. The Kurokawa et al reference is directed to expression of nerve growth factor and enhancement of disulfide bond formation. This reference does not teach or suggest a polynucleotide that encodes a prokaryotic secretion signal sequence and a polynucleotide encoding an intact antibody comprising a variant heavy chain, wherein the variant heavy chain comprises a variant hinge region which does not form inter-heavy chain disulfide linkages. The Kurokawa reference describes enhancing the disulfide bond formation.

In view of the remarks above, the rejection under 35 U.S.C. § 103(a) is believed to be overcome. Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

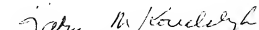
In view of the above amendments and remarks, Applicants respectfully request a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

Respectfully submitted,

23552
PATENT TRADEMARK OFFICE

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

Date: Oct. 19 2007



Katherine M. Kowalchyk
Reg. No. 36,848

RNase P RNA did not serve as an RNA ligase substrate. The oxidized RNA bonds contained less than 2 percent of the [^{32}P]pCp incorporated into the unmodified RNA, as determined by scintillation counting of gel slices and remained intact, as judged by silver staining (*4*, *5*). Incubation of oxidized RNase P RNA in the high salt buffer of the RNase P assay had no effect on its later ability to serve as an RNA ligase substrate, demonstrating that the oxidized terminus is stable under the unusual ionic conditions of the assay. Furthermore, periodate-oxidized precursor tRNA could serve equally well as substrate for either native or oxidized RNase P RNA (*5*). Thus, the 3′ termini of neither the RNase P RNA nor its substrate are required for accurate processing. The mechanism of the RNase P reaction is different from those that occur during intron excision from *Tetrahymena* rRNA.

Since the 3′-OH of the RNase P RNA is not the initiating nucleophile in the RNase P processing reaction, it is likely that a hydroxyl group from water serves that role. The RNase P reaction had a significant dependence on hydroxide concentration (Fig. 1C) with an optimum at *p*H 8 to 8.5. The *p*H at the half-maximum reaction rate does not correlate with any of the reported *p*K values for nucleic acid ionizable groups, consistent with dependence on hydroxide concentration. Above an approximate *p*H of 9, all activity was lost, probably because of denaturation of the catalyst or substrate RNA's due to deprotonation of U-N3 (at *p*H 9 to 9.5).

Reactions involving hydroxide attack on phosphate esters are commonly catalyzed by proteins. The amino acid side chains may provide proton exchange mechanics to activate a water molecule positioned to attack the esterified phosphorus (Fig. 2). We envisage that the precursor tRNA substrate binds to the surface of the RNase P RNA in a manner analogous to the binding of a substrate to a protein surface, and that nucleic acid groups manipulate the reactants. A hydrated Mg^{+2}-coordinate complex offers attractive geometry for the reaction (*6*), but the actual catalysis must derive from the RNase P RNA. The proton donor-acceptor roles might be provided by nucleoside base tautomeric transitions, nonterminal 2-OH groups, or, conceivably, internucleotide phosphates with high *p*K values. The delineation of the active site, the identification of chemically important groups, and the roles of high concentrations of mono- and divalent cations required for the RNA reaction remain to be investigated.

References and Notes

1. K. Kruger, P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gottschling, T. R. Cech, *Cell* **31**, 147 (1982).
2. C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, *ibid.* **35**, 849 (1983).
3. C. Guerrier-Takada and S. Altman, *Science* **223**, 285 (1984).
4. B. R. Oakley, D. R. Kirsch, N. R. Morris, *Anal. Biochem.* **105**, 361 (1980).
5. T. L. Marsh and N. R. Pace, unpublished data.
6. K. Haydock and L. C. Allen, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
7. S. H. Leppla, B. Bjoraker, R. M. Bock, *Methods Enzymol.* **12**, 236 (1968). Briefly, approximately 1.0 to 1.5 μg of RNase P RNA in H_2O was mixed with a 100-fold molar excess of sodium metaperiodate. A subcatalytic amount of 3′-labeled RNase P RNA was added to all reactions, or to control reactions run in parallel, as a means of assessing recovery. The periodate was added under darkroom safety lights and the reaction mixture was incubated for 1 hour at room temperature in the dark. Then the 15-μl reaction mixture was diluted 1:10 with H_2O, sodium acetate (*p*H 5.4) was added to a final concentration of 0.2*M*, and the RNA was precipitated with three volumes of ethanol. The RNase P RNA was centrifuged (Eppendorf), dried briefly under vacuum, and resuspended in 15 μl of H_2O. The resuspended RNA was divided into two portions; one was tested for RNase P processing activity and the other for effectiveness as a substrate in an RNA ligase reaction.
8. D. A. Stahl *et al.*, *Nucleic Acids Res.* **9**, 6129 (1981).
9. We thank B. Pace and C. Reich for assistance. Supported by NIH research grant GM34527 (N.P.R.).

6 December 1984; accepted 29 March 1985

Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G_1 Fragments

Abstract. *Preparation of bispecific antibodies by the chemical reassociation of monovalent fragments derived from monoclonal mouse immunoglobulin G_1 is inefficient because of side reactions during reoxidation of the multiple disulfide bonds linking the heavy chains. These side reactions can be avoided by using specific dithiol complexing agents such as arsenite and effecting disulfide formation with a thiol activating agent such as 5,5′-dithiobis(2-nitrobenzoic acid). In this way bispecific antibodies were obtained in high yield and free of monospecific contaminants from monoclonal mouse immunoglobulin G_1 fragments. The bispecific antibodies were used as agents for the selective immobilization of enzymes.*

MAUREEN BRENNAN
Department of Fine Structure, Boston Biomedical Research Institute, Boston, Massachusetts 02114
PETER F. DAVISON
Department of Fine Structure, Boston Biomedical Research Institute, and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02115
HENRY PAULUS
Department of Metabolic Regulation, Boston Biomedical Research Institute, and Department of Biological Chemistry, Harvard Medical School

Bispecific antibodies, hybrid immunoglobulins with two different antigen-binding sites, have been prepared from polyclonal rabbit immunoglobulins (*1*-*3*). They have many potential uses, ranging from immunodiagnostic procedures to targeted delivery of drugs. Nevertheless, the applications of bispecific antibodies would be considerably enhanced if they could be derived from monoclonal antibodies. The preparation of bispecific monoclonal antibodies by fusion of antibody-producing cells was recently described by Milstein and Cuello (*4*). Although their procedure produces a mixture of hybrid antibodies with various assortments of chains, which must be fractionated to yield the desired bispecific molecules, they suggested that it is superior to a chemical reconstitution approach on account of certain technical problems (*4*). These problems include the need to dissociate immunoglobulins into half-molecules without damaging the antigen-binding sites and to reform three disulfide bonds linking the heavy chains, characteristic of mouse immunoglobulins, without allowing interfering side reactions, such as formation of disulfide bonds within chains. Nevertheless, it has been possible to obtain hybrid monoclonal antibodies in low yields (*5*). We report here a chemical procedure for preparing bispecific antibody fragments from monoclonal mouse immunoglobulin G_1 (IgG_1), a procedure that avoids these problems and generates the desired bispecific reagent in high yield as the only product, obviating the need for further purification.

To dissociate reduced immunoglobulin half-molecules under mild conditions, we used the method of Nisonoff and Mandy (*1*), who removed the Fc′ portion of rabbit IgG by limited pepsin hydrolysis to yield $F(ab')_2$. The cleavage of monoclonal mouse IgG_1 with pepsin to yield $F(ab')_2$ proceeded readily, provided that the reaction was carried out at a slightly lower *p*H than that with rabbit IgG (*6*, *7*). About 80 percent of the monoclonal IgG_1's were converted to $F(ab')_2$ with a good yield (75 to 95 percent) by incubation with pepsin (2 percent by weight) in 0.1*M* sodium acetate (*p*H 4.2) for 18 hours at 37°C. On reduc-

tion of $F(ab')_2$ with 1 m*M* 2-mercaptoethylamine in 0.1*M* sodium phosphate (*p*H 6.8) and 1 m*M* EDTA for 18 hours at 25°C, complete conversion to Fab' was observed.

Our attempts and those of others (*5*) to regenerate $F(ab')_2$ from Fab' prepared as described above gave yields that were generally poor compared to those obtained with rabbit immunoglobulin fragments (*1–3*). This problem was probably related to the three disulfide bonds that bridge the heavy chains in mouse IgG (*8*) (in contrast to a single such bond in rabbit IgG), so that dimerization, which involves formation of disulfides between chains, must compete with disulfide formation within chains. Our strategy for avoiding this problem involved two major modifications of the procedure of Nisonoff and Mandy (*1*). The first consisted of carrying out the reduction of $F(ab')_2$ in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols (*9*) and impede intramolecular disulfide formation. The second involved activating the thiols of one of the Fab' preparations as the thionitrobenzoate (TNB) derivative (*10*). The reaction scheme (Fig. 1) consists of the reduction of two different $F(ab')_2$ fragments with 2-mercaptoethylamine in the presence of sodium arsenite and their conversion to the TNB derivatives by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with 2-mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the hybrid dimer.

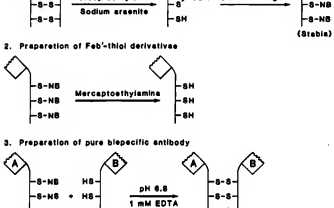


Fig. 1. Reaction sequence for the preparation of bispecific antibodies from monoclonal mouse IgG_1 fragments. Only the hinge region of the Fab' fragments is shown.

The efficacy of sodium arsenite in preventing intrachain disulfide bonds was demonstrated by titration with Ellman's reagent. When $F(ab')_2$ was reduced in the absence of arsenite, monoclonal mouse Fab' and rabbit Fab' bound nearly the same amount of TNB, but mouse $F(ab')_2$ reduced in the presence of arsenite bound approximately three times more TNB. The use of Ellman's reagent (*10*) as a thiol activating agent had four important advantages. First, the Fab'-TNB derivative was a relatively stable compound, and thus a convenient intermediate, which could be stored at 4°C for several days with only slight deterioration. Second, Fab'-TNB was also a convenient source of Fab'-thiol, which could be generated by brief exposure to 2-mercaptoethylamine. Third, the reaction of a Fab'-TNB with a Fab'-thiol led to the bispecific $F(ab')_2$ as the sole product, obviating the need for separation from symmetrical dimeric products. Finally, thiol activation with Ellman's reagent allowed bispecific antibody formation in the absence of net thiol oxidation. EDTA could thus be present at all steps to prevent heavy metal–catalyzed disulfide formation, ensuring that—even without exclusion of O_2 from the atmosphere and solvents—the Fab'-thiol reactant did not self-dimerize.

An example of the formation of a bispecific antibody from an anti-avidin Fab' and an anti-luciferase Fab' is shown in Fig. 2, the reaction being monitored by high-performance gel exclusion chromatography. Under the coupling conditions used, the activated anti-avidin Fab' and the thiol form of anti-luciferase Fab' alone formed only trace amounts of $F(ab')_2$, whereas an equimolar mixture formed $F(ab')_2$ with a yield of 70 percent. (The small amount of IgG in each of the samples served as an internal chromatographic standard.) The absence of dimer

Table 1. Enzyme immobilization by bispecific antibodies. Biotinylated regenerated cellulose membranes (4.8-mm α-Metricel disks; Gelman) were prepared by CNBr activation (*16*) and substitution with biocytin (*17*). They were then incubated with or without avidin (5 μg) in 50 μl of buffer G (10 m*M* potassium phosphate and 0.6*M* NaCl; *p*H 7.4) containing bovine serum albumin (10 mg/ml) for 1 hour at 4°C. After brief washing with buffer G containing 0.1 percent Tween 20, the membranes were incubated for 2 hours at 4°C in 100 μl of buffer G containing 1 percent bovine serum albumin and the bispecific antibodies were directed against different epitopes of *E. coli* β-galactosidase (βG). After being washed five times with 0.5 ml of buffer G containing 0.1 percent Tween 20, the membranes were assayed for βG activity (*18*) or horseradish peroxidase (HRP) activity (*19*) with *o*-nitrophenylgalactoside or *o*-phenylenediamine, respectively, as chromogenic substrates, by measuring absorbance at 420 or 490 nm.

Incubation mixture	Enzyme measured	Enzyme activity immobilized
Experiment 1		
Complete*	βG	1.098†
Without avidin	βG	0.015
Without anti–avidin:βG $F(ab')_2$	βG	0.030
Experiment 2		
Complete‡	HRP	3.0§
Without avidin	HRP	0.083
Without anti–avidin:HRP $F(ab')_2$	HRP	0.114
Experiment 3		
Complete‖	HRP	0.961¶
Without avidin	HRP	0.082
Without anti–avidin:βG $F(ab')_2$	HRP	0.167
Without βG	HRP	0.107
Without anti–βG:HRP $F(ab')_2$	HRP	0.080

*Biotinylated α-Metricel disk, avidin (5 μg), anti-avidin:βG $F(ab')_2$ (0.5 μg), and *E. coli* βG (0.5 μg). †Twenty percent of βG input. ‡Biotinylated α-Metricel disk, avidin (5 μg), anti-avidin:HRP $F(ab')_2$ (0.5 μg), and HRP (1 μg). §One percent of peroxidase input. ‖Biotinylated α-Metricel disk, avidin (5 μg), anti-avidin:βG $F(ab')_2$ (0.5 μg), βG (0.5 μg), anti-βG:HRP $F(ab')_2$ (0.5 μg), and HRP (1 μg). ¶Two-tenths of 1 percent of peroxidase input.

formation when either of the reaction partners was absent suggested that the dimer formed in the complete mixture represented exclusively pure bispecific antibody. Pure product can thus be conveniently obtained by subjecting the reaction mixture to high-performance gel exclusion chromatography and collecting the $F(ab')_2$ fraction (*11*). The yields of $F(ab')_2$ obtained with many combinations of different Fab' pieces were generally 50 to 70 percent. No attempt was made to optimize yield by modifying the reaction conditions.

Besides yield, an important consideration in evaluating our synthetic procedure is product quality. To determine whether the chemical manipulations caused irreversible alterations in the antigen-binding site, we carried a monoclonal anti-β-galactosidase $F(ab')_2$ through the procedure, thus presumably regenerating the starting material. We compared the immunoreactivity of the original and reconstituted $F(ab')_2$ in an enzyme-linked immunoassay against β-galactosidase and found no significant difference. This suggested that there was no substantial alteration or loss of antigen-binding sites. On the other hand, titration with [^{14}C]*N*-ethylmaleimide occasionally showed the presence of 0.1 to 0.2 mol of residual-free thiol per mole of $F(ab')_2$, indicating that not all disulfides were completely reformed. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that about 10 percent of the product migrated slightly more rapidly than $F(ab')_2$, accompanied by a corresponding amount of free light chains. These observations suggested that our procedure led to some reduction and incomplete reoxidation of the disulfide bond between heavy and light chains, without significantly affecting the integrity of the antigen-binding site.

To ascertain whether the reaction products were indeed bispecific antibodies, we carried out our procedure with monoclonal Fab' against avidin on the one hand and against *Escherichia coli* β-galactosidase or horseradish peroxidase on the other. We postulated that the resulting bispecific antibodies would be able to act as linkers for immobilization of the enzymes on a biotin-substituted matrix in the presence of avidin. As shown in Table 1, the avidin-dependent binding of β-galactosidase and peroxidase to biotinylated regenerated cellulose membranes occurred in the presence of the corresponding bispecific $F(ab')_2$. The ability of bispecific antibodies to act as heterobifunctional protein cross-linkers was even more strikingly illustrated by the immobilization of peroxidase on biotin-substituted cellulose in the presence of two bispecific antibodies, one with binding sites for avidin and β-galactosidase, the other for β-galactosidase and peroxidase, as well as both avidin and β-galactosidase. This illustrates the potential use of bispecific antibodies for the highly specific coimmobilization of multiple enzymes (*12*). Such complexes are self-assembling and functional enzyme sequences can be generated, even from impure enzyme mixtures.

In conclusion, we have described a rapid and efficient method for the preparation of bispecific antibodies from monoclonal mouse IgG_1. It differs from the cell fusion approach (*4*) by producing $F(ab')_2$ instead of intact IgG but gives pure products in higher yields with greater ease. Whereas cell fusion is laborintensive and may not always succeed with the hybridoma pair of choice, our chemical procedure allows a single operator to prepare many different bispecific antibodies in less than 1 week, the only experimental limitation being the susceptibility of the monoclonal antibodies to selective cleavage by pepsin. Most important, many applications of bispecific antibodies require pure preparations (*12*), which are obtained directly by our chemical reconstitution method. In contrast, the complex antibody mixtures produced by hybrid hybridomas must be purified either by difficult chromatographic procedures or by immunoaffinity fractionation involving drastic desorption conditions that are likely to cause some irreversible denaturation.

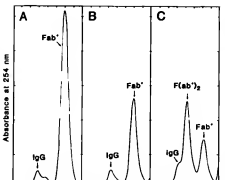


Fig. 2. Formation of bispecific antibodies. BALB/c mice were immunized by repeated injections of avidin or *Photinus* luciferase and their spleen cells were fused by conventional methods (*13*) with P3-NS1/1-Ag4 myeloma cells obtained from the Cell Distribution Center, Salk Institute. Hybrid cells were selected in hypoxanthine, aminopterin, and thymidine medium (*14*) and screened by enzyme-linked immunoassay, and selected colonies were cloned by limiting dilution on thymocyte feeder layers. Hybridomas secreting IgG_1 were grown as ascites tumors. The IgG_1 in the fluid was purified by salting out and DEAE chromatography and digested with pepsin as described in the text. The $F(ab')_2$ fragments were purified by high-performance liquid chromatography (HPLC) on a TSK-3000SW column in 0.1*M* sodium phosphate (*p*H 6.8) and then reduced overnight (3 mg/ml; 25°C) with 1 m*M* 2-mercaptoethylamine in the same buffer with 1 m*M* EDTA and 10 m*M* sodium arsenite. Solid Ellman's reagent was added to a concentration of 5 m*M* and, after 3 hours at 25°C, the excess reagent was removed by centrifugal gel filtration on Sephadex G-25 (*15*). The thiol form of the anti-luciferase Fab' was regenerated by a 30-minute treatment with 10 m*M* mercaptoethylamine followed by centrifugal gel filtration. Although the reactions described were nearly quantitative, the yield at this stage was about 75 percent because of mechanical losses during the gel filtration procedures. Shown are (*A*) the TNB derivative of anti-avidin Fab'(0.19 mg), (B) the thiol form of anti-luciferase Fab' (0.19 mg), and (C) a mixture of these, at a protein concentration of 2.4 mg/ml, incubated for 16 hours at 25°C in 0.1*M* sodium phosphate (*p*H 6.8) and 1 m*M* EDTA, all treated with 5 m*M* Ellman's reagent to dissociate any noncovalent aggregates and subjected to HPLC as described above. The starting materials showed no change during the incubation period, whereas their mixture was converted to $F(ab')_2$ with a yield of about 70 percent. Bispecific antibody was obtained with a yield of 48 percent after purification by HPLC.

References and Notes

1. A. Nisonoff and W. J. Mandy, *Nature (London)* **194**, 355 (1962).
2. U. Hammerling, T. Aoki, E. deHarven, E. A. Boyce, C. J. Ökol, *J. Exp. Med.* **128**, 1461 (1968).
3. V. Raso and T. Griffin, *Cancer Res.* **41**, 2073 (1981).
4. C. Milstein and A. C. Cuello, *Nature (London)* **305**, 537 (1983).
5. P. Parham, *Hum. Immunol.*, in press.
6. E. Lamoyi and A. Nisonoff, *J. Immunol. Methods* **56**, 235 (1983).
7. P. Parham, *J. Immunol.* **131**, 2895 (1983).
8. J. Svasti and C. Milstein, *Biochem. J.* **126**, 837 (1972).
9. J. L. Webb, *Enzymes and Metabolic Inhibitors* (Academic Press, New York, 1960), vol. 3, pp. 595–79.
10. G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
11. A portion of the residual Fab' formed noncovalent aggregates. We found it expedient to supplement the mixture with 5 m*M* Ellman's reagent before chromatography in order to dissociate any noncovalent aggregates. This treatment had no effect on the disulfide-bonded $F(ab')_2$.
12. H. Paulus, U.S. patent 4,444,878 (1984).
13. G. Galfre *et al.*, *Nature (London)* **266**, 550 (1977).
14. J. W. Littlefield, *Science* **145**, 709 (1964).
15. A. J. Parkinson, E. N. Scott, H. G. Muchmore, *Anal. Biochem.* **118**, 401 (1981).
16. J. Kohn and M. Wilchek, *Biochem. Biophys. Res. Commun.* **107**, 878 (1982).
17. E. Bayer and M. Wilchek, *Methods Enzymol.* **34**, 265 (1974).
18. A. R. Frackelton and B. Rotman, *J. Biol. Chem.* **255**, 5286 (1980).
19. E. Engvall, *Methods Enzymol.* **70**, 419 (1980).

20. We thank C. Brown, J. Thaxter, and P. Yelick for expert technical assistance and P. Parham for his results. Supported by a grant from Polaroid Corporation.

28 December 1984; accepted 4 March 1985

JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2465–2471
0021-9193/97/$04.00+0

Vol. 179, No. 8

MINIREVIEW

Protein Folding in the Bacterial Periplasm

DOMINIQUE MISSIAKAS[1] AND SATISH RAINA[2]*

Centre National de Recherche Scientifique, LIDSM-CBBM, 13402 Marseille Cedex 20, France,[1] and Département de Biochimie Médicale, Centre Médical Universitaire, 1211 Genève 4, Switzerland[2]

INTRODUCTION

The discovery of protein chaperones is a hallmark in the study of protein folding in vivo. The problems inherent to aggregation-prone nascent polypeptide chains are mostly alleviated by two families of chaperones, Hsp60 and Hsp70, also known as the GroEL and DnaK proteins, respectively, in *Escherichia coli* (20, 21). These chaperones stabilize unstable conformers of proteins during folding, translocation through lipid barriers, and assembly (29). Chaperones such as GroEL contain a large cavity in their structure where they can accommodate loosely defined substrates such as "molten globules." The synergistic action of GroEL and GroES leads to either the release of a fully folded protein or to a structural intermediate that completes folding after release (51, 80). In some cases, multiple binding and release of a partly folded polypeptide may be required to achieve correct folding (51). At high temperatures, unfolding is a major problem resulting in a greater need for chaperones to prevent drastic protein aggregation and to promote disaggregation or even proteolysis of damaged proteins. It is quite remarkable that many chaperones and proteases are expressed at a higher level at elevated temperatures and especially following heat shock. Most of them are encoded by heat shock-regulated genes (26, 56).

The folding of proteins residing in or transiting through the periplasm of gram-negative bacteria is less well understood. The periplasm is much more vulnerable to changes in the external environment, e.g., changes in pH, temperature, and osmolarity. This vulnerability is particularly severe in bacteria with leaky outer membranes. So far, none of the classical molecular chaperones has been identified in the periplasm, probably because ATP, which is crucial for the activity of the Hsp60 and Hsp70 families, is absent. Unlike cytoplasmic proteins, exported proteins tend to contain disulfide bonds which contribute to their stability and, in some cases, are essential for their catalytic activity. This calls for maintenance of the periplasm in a relatively more oxidizing state.

Accessory proteins in the periplasm have been identified as folding catalysts which contribute rather differently from the chaperones to the protein folding reaction. Folding catalysts accelerate only a specific rate-limiting step of the folding reaction. Among them, the protein disulfide isomerases (PDI) perform thiol-disulfide exchanges, which are obligatory reactions during disulfide bond formation or rearrangement. The second type of slow conformational rearrangement, caused by isomerization around the Xaa-Pro peptidyl bond, is catalyzed by the peptidyl prolyl isomerases (PPI). The discovery and activity of the folding enzymes operating in the bacterial periplasm are the topics of this review.

* Corresponding author. Mailing address: Département de Biochimie Médicale, Centre Médical Universitaire, 1 Rue Michel-Servet, 1211 Genève 4, Switzerland. Phone: 41 22 702 55 11. Fax: 41 22 702 55 02. E-mail: raina@cmu.unige.ch.

WHY DISULFIDE BONDS AND WHERE? OXIDATION OF TRANSPORTED PROTEINS

Disulfide bonds usually contribute to the stabilization of a folded protein conformation (11). Thus, reducing such disulfide bonds and breaking the covalent linkage will induce unfolding of the protein, even in a buffer devoid of any sort of denaturant. Proteins exist, such as β-lactamase or α-lactalbumin, which do not fall apart upon reduction of their disulfide bonds. Even so, disulfide bridges which do not contribute directly to protein stability define a particular conformation of the protein. Introducing the wrong cysteine pairing often leads to an unstable or inactive polypeptide because of a wrong fold.

In vitro, disulfide bond formation is an extremely slow reaction and can take from hours to days. However, such reactions are fast in vivo, where most of the disulfide-bonded proteins are exported proteins. As a rule, their oxidation does not occur in the compartments where they are synthesized. Because polypeptides destined to leave the cytoplasm of bacteria or the cytosol of eukaryotic cells must remain in an extended conformation to be properly translocated, oxidation in such compartments should be avoided. Hence, disulfide bonds are formed in the endoplasmic reticulum (ER) of eukaryotes and in the periplasm of bacteria.

Disulfide formation in the cytosol of eukaryotes is largely prevented by the reduced environment it provides with a ratio of reduced/oxidized glutathione (GSH/GSSG) estimated to be between 30:1 and 100:1 (18). In the ER, the redox environment is "buffered" due to the high concentration of both reduced and oxidized glutathione. It is of importance that an almost constant ratio of about 3:1 is maintained between these two species (33), although the mechanism by which this ratio is maintained is unknown. Such a situation favors the thermodynamic formation of disulfide bonds trapped in folded or almost folded proteins. About 30 years ago, an activity able to reactivate reduced and denatured RNase A was isolated (24). Its ability to catalyze the reformation of the correct intramolecular disulfide bonds in scrambled RNase A by using the proper redox buffer (GSH/GSSG) was the reason why such an enzyme was named PDI (78).

Disulfide bond catalysis is slightly more complicated in the periplasm of gram-negative bacteria than in the ER. The periplasm is separated from the extracellular environment by a porous membrane which allows passive diffusion of small molecules. Variations in the medium composition do not, therefore, favor the existence of a defined redox potential. However, it would be premature to rule out the existence of a redox gradient actively maintained by the cell among the cytoplasm, periplasm, and medium. At least, it seems that in the cytoplasm

a reducing balance is established with three glutaredoxin activities (2), as well as the thioredoxin and thioredoxin reductase system (71).

GENETIC ISOLATION OF *dsb* AND RELATED GENES

The first hint about a gene(s) encoding PDI-like activities was rather fortuitous. Genetic screens set up to isolate mutants defective in the insertion of proteins into the inner membrane identified a gene designated *dsbA* (4). Mutations in *dsbA* are highly pleiotropic, and the designation *dsb*, for disulfide bond formation, was given after deducing that the gene encodes a periplasmic thiol:disulfide oxidoreductase (4). In these studies, the model protein was a hybrid consisting of the membrane protein MalF and β-galactosidase (β-gal). This hybrid protein confers a Lac$^+$ phenotype if it remains in the cytoplasm. It turned out that in a *dsbA* mutant, β-gal presumably remained reduced and as a consequence unfolded, allowing the hybrid MalF–β-gal to slide back into the cytoplasm. Later, the same genetic approach was used to identify a second gene encoding a putative thiol:disulfide oxidoreductase, designated *dsbB* (3). Hence, deciphering of the function of the *dsbA* gene opened the door to a rapidly expanding field with respect to protein folding in the periplasm.

The *dsbA* gene was also identified by two other independent genetic approaches. The first one consisted of looking for mutations leading to decreased alkaline phosphatase (AP) activity. AP is a periplasmic enzyme which contains two disulfide bonds in its folded conformation. Mutations in the *dsbA* gene were found to block AP activity (37). The second genetic search was more direct, since it was aimed at isolating thiol: disulfide oxidoreductase activities by looking for mutants which were hypersensitive to dithiothreitol (DTT), a strong reducing agent. Addition of DTT primarily offsets the oxidizing balance of the periplasm, and mutants unable to cope with small variations in redox potential could be obtained. Mutations in the *dsbA*, *dsbB*, and *trxB* genes, among others, were obtained (61). Finally, this general approach was also used to clone genes which in multicopy confer resistance to sublethal concentrations of DTT. Resistance to high DTT concentrations could be conferred by the *dsbB* gene (61). This allowed direct selection for its cloning.

It soon became clear that the pleiotropic phenotype of mutations in either *dsbA* or *dsbB* could be attributed to folding defects of various disulfide bond-containing proteins. For instance, *dsbA* and *dsbB* mutations were also isolated because they blocked cellular pathways or activities depending solely on the presence of one disulfide bond-containing protein. Hence, *dsbB* mutants were isolated because they conferred a motility defect on *E. coli* due to accumulation of the reduced P-ring protein forming the flagellar basal body (14). This nonmotile phenotype is also observed in bacteria carrying mutations in *dsbA* (14). In *Vibrio cholerae*, biogenesis of the toxin-coregulated colonization pilus was found to require the *tcpG* gene product, a protein 40% identical to *E. coli*'s DsbA (66). Mutations in *tcpG* were independently found to be responsible for the defective production of the enterotoxin B subunit (EtxB), another disulfide bond-containing protein whose toxicity was highly reduced in *tcpG* mutants (84, 85). Another DsbA homolog, Por (45% identical to the *E. coli* DsbA protein), was identified in *Haemophilus influenzae* because mutations in the Por-encoding gene impaired competence-induced DNA uptake in this bacterium, presumably because this process involves outer membrane proteins (OMPs) with disulfide bonds in their active state (76). The list is not exhaustive, and there are many other cases in which mutations in *dsbA* have been found to block the folding of secreted proteins and, in some cases, attenuate the virulence of certain pathogenic bacteria (1, 7, 35, 64, 83).

IDENTIFICATION OF THIOL:DISULFIDE OXIDOREDUCTASES OTHER THAN DsbA AND DsbB

A search for suppressors able to reverse the DTT sensitivity of *dsbA* mutants revealed further prokaryotic PDI-like activities, designated DsbC, DsbD, and DsbE (see Fig. 3) (58–60). These enzymes seem to have either thiol:disulfide isomerase (DsbC) or thiol:disulfide reductase (DsbD and DsbE) activity. Hence, the hypersensitivity to DTT exhibited by *dsbA* mutants can be alleviated in two ways: either by overexpressing *dsbC* (60) or by null mutations in either *dsbD* (59) or *dsbE*. DsbC was also identified independently in *Erwinia chrysanthemi* by Shevchik and colleagues (73), who used *E. chrysanthemi* DNA libraries to complement an *E. coli dsbA* mutant.

DsbC, like DsbA, is a periplasmic protein. Its multicopy effect on a *dsbA* mutant suggests some overlap of functions between the two proteins. DsbD is a membrane-bound protein. Given the context of its isolation, it has been proposed that since DsbA acts as a thiol:disulfide oxidase, DsbD should encode the opposite activity, that of a thiol:disulfide reductase. This assumes that the suppression effect of a *dsbD* null mutation in *dsbA* null mutant bacteria occurs by setting a better oxidizing balance in the periplasm. In fact, DsbC accumulates mostly in its oxidized form in such a background and thus may actively substitute for the lack of DsbA (59). DsbD is also referred to as CutA2 (17) or DipZ (13). The *dsbD* transcription start site is indeed part of the *cutA* locus. The *cutA* locus has been implicated in copper homeostasis, since such mutant bacteria are sensitive to Cu^{2+} ions (17). DipZ, or the disulfide isomerase protein-like domain, was discovered because it is involved in the biogenesis of periplasmic *c*-type cytochromes (13; see below). Similarly, mutations in *dsbE* were found to partially restore the DTT sensitivity observed with either *dsbA* or *dsbB* mutant bacteria (58). However, a null mutation in *dsbE* does not confer sensitivity to reduced DTT by itself. On the contrary, *dsbE* mutant bacteria are sensitive to oxidized DTT. Hence, the suppressing effect of a *dsbE* null mutation argues that DsbE might act in vivo more like a thiol:disulfide reductase than a thiol:disulfide oxidase. These results are consistent with its biochemical properties (see below).

Dsb PROTEINS BELONG TO THE THIOREDOXIN SUPERFAMILY—THE CHEMISTRY OF THIOL-DISULFIDE EXCHANGE REACTIONS

Unlike PDI, Dsb proteins do not share overall sequence homology with thioredoxins, yet all of these proteins share at least one similar active site, which is Cys-X-X-Cys (Fig. 1). A first alignment of the predicted secondary structures of DsbA and PDI and the known thioredoxin structure revealed the presence of a common fold (15). This has been confirmed with the resolution of DsbA's three-dimensional structure. Half of the DsbA protein (21 kDa) is structured as a domain superimposable on the thioredoxin fold (about 10 kDa in mass) with an additional globular domain. The packing between the two domains results in a cleft which was proposed to be the substrate acceptor site (50). The Cys^{30}-Pro-His-Cys^{33} motif (Fig. 1), as in thioredoxins, lies at the end of an α-helix (50). Three-dimensional structures are not available for PDI and DsbC, although crystals have been obtained for DsbC (72). Both of these proteins are large enough to contain a thioredoxin fold. In the case of PDI, which contains two Cys-X-X-Cys motifs,

DsbA	Cys-Pro-His-Cys
DsbB	Cys-Val-Leu-Cys
DsbC	Cys-Gly-Tyr-Cys
DsbD	Cys-Val-Ala-Cys
DsbE	Cys-Pro-Thr-Cys
Thioredoxin	Cys-Gly-Pro-Cys
PDI	Cys-Gly-His-Cys

FIG. 1. The thiol-disulfide active sites of members of the thioredoxin superfamily.

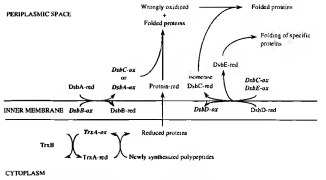


FIG. 3. Model of disulfide formation catalyzed by the various Dsb enzymes in the periplasm of *E. coli*.

nuclear magnetic resonance characterization of the N-terminal thioredoxin-like domain has confirmed its modular structure (39).

Among Dsbs, the membrane-bound DsbD protein presents an exception in terms of its homology with PDI. DsbD contains a 16-kDa C-terminal domain, highly hydrophilic, facing the periplasm (53, 59). This domain is about 40 to 45% identical to the thioredoxin-like domain found in eukaryotic PDIs. There are two Cys-X-X-Cys motifs in the DsbD sequence, but the Cys^{403}-Val-Ala-Cys^{406} redox site located in the 16-kDa C-terminal domain has been shown to carry the thiol-disulfide exchange reactions essential for protein folding (59).

The thiol-disulfide exchange reaction has been exhaustively characterized with thioredoxin and glutaredoxin (30). Between the most N-terminal Cys residue in the active site of thiol: disulfide oxidoreductases and the substrate, a mixed disulfide is formed as an obligatory intermediate. Such an intermediate is rapidly resolved by the second cysteine present in the active site of the catalyst (Fig. 2). For example, a mixed disulfide with DsbA reacts with an external thiol 10^3-fold faster than a normal mixed disulfide (81, 88). The equilibrium constant for the formation of the disulfide active site as measured by interchange with glutathione is 80 μM for DsbA (88) and 200 μM for DsbC (86). As a comparison, a 10 M equilibrium constant has been measured for the formation of a disulfide in the active site of thioredoxin (31) and values of up to 10^5 M can be measured for normal stabilizing bonds in folded proteins (12). Hence, the disulfide bonds in the active sites of DsbA and DsbC are very unstable. DsbA is a much better oxidant than thioredoxin (the standard redox potentials for DsbA, PDI, and thioredoxin are, respectively, 0.089 V [81], −0.11 V [28], and 0.27 V [43] at pH 7 and 30°C). Overall, the reduced forms of the DsbA and DsbC proteins (82, 86, 87) are more stable than their oxidized forms. This destabilizing effect determines the redox properties of DsbA and DsbC. Normally, disulfide bonds have stabilizing effects on folded conformations and also on thiol:disulfide reductases like thioredoxin (38). It has been suggested that the nature of the two amino acids in the active-site sequence of the thioredoxin-like motif (Fig. 1) could be a key determinant of the redox properties of the oxidoreductases. As an example, the Pro-to-His substitution in *E. coli*'s thioredoxin active site (which makes it identical to the active-site sequence of PDI) results in a change of the redox potential value from −0.27 to −0.235 V (43) and a better ability to reactivate scrambled RNase A (49). Similarly, swapping DsbA's motif for the thioredoxin motif results in a DsbA protein with the thiol:disulfide chemistry of thioredoxin (23). It seems that the role of the two residues within DsbA's Cys^{30}-Pro-His-Cys^{33} motif is to specifically lower the pKa of Cys^{30}, which is critical for the high oxidizing capacity of DsbA (25, 63).

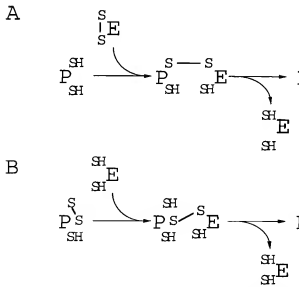


FIG. 2. Formation of mixed disulfide species consisting of a polypeptide (P) and a thiol:disulfide oxidoreductase (E). (A) Oxidation of a polypeptide, the enzyme being the direct oxidant. (B) Isomerization reaction.

Comparative analyses of the kinetic properties of the DsbA and DsbC active sites have led to the suggestion that DsbA is more efficient at transferring its disulfide bond to other proteins (88; Fig. 2A), whereas DsbC, like PDI, is more efficient at catalyzing disulfide rearrangements (86; Fig. 2B). Like PDI, DsbC is also able to bypass disulfide rearrangements in quasi-native species of bovine pancreatic trypsin inhibitor (BPTI) by directly incorporating the missing disulfide, suggesting a possible unfoldase activity. BPTI contains three disulfides in the native state, and their formation reflects the kinetics of folding of the protein. The fact that DsbC, unlike DsbA, is a dimer might be a clue to its more PDI-like behavior, since active monomeric PDI contains two redox-active sites.

BIOLOGICAL ACTIVITY OF THE Dsb PROTEINS

The DsbA and DsbB couple. In vivo evidence suggest that the DsbA and DsbB proteins are part of the same pathway and act in concert to oxidize many transported proteins (Fig. 3). The first evidence came from the observation that DsbA ac-

cumulates mostly in the reduced form in the periplasm of *dsbB* mutant bacteria, whereas in wild-type bacteria both reduced and oxidized species are present (3, 60). A model suggesting that DsbB could reoxidize the reduced DsbA protein, thereby recycling an active DsbA thiol:disulfide oxidase, was proposed (3). Because of the thiol:disulfide chemistry, this model implies that a mixed disulfide containing of DsbA and DsbB should be formed via the cysteines of their active sites. Previous experiments had shown that a mixed disulfide could readily be trapped in vivo between glutaredoxin (mutation of Cys-14 to Ser) and glutathione in the cytoplasm, provided that only the reactive, first cysteine of each dithiol motif was present (9). A mixed disulfide, DsbA-Cys^{30}-Cys^{104}-DsbB, could be isolated (27, 41). It was further proved that among the four periplasmic cysteines found to be essential for DsbB activity (36), Cys^{104} forms a reversible disulfide bond with Cys^{130} (40). This bond is the one transferred to DsbA. The Cys^{104}-Cys^{130} linkage is reoxidized intramolecularly by the N-terminally located Cys^{41}-Val-Leu-Cys^{44} thioredoxin-like motif of the DsbB protein (40).

Rat PDI secreted in the periplasmic space of *E. coli* is able to complement several phenotypes exhibited by *dsbA* mutants (65). Moreover, it has been shown that disulfide bond formation catalyzed by rat PDI is dependent on the presence of the *dsbB* gene. This suggests that the reoxidation of the eukaryotic enzyme, which in the ER is dependent on the GSH/GSSG components, proceeds in the periplasm by direct interaction with the bacterial redox proteins (65).

Thiol:disulfide isomerase and reductase activities in the periplasm: the DsbC, DsbD, and DsbE proteins. DsbC was originally isolated because it could, when expressed from a multicopy plasmid, reverse most of the phenotypic defects caused by the lack of a functional DsbA protein in vivo (such as lack of AP activity or motility) (60, 72). In vivo, it appears that DsbC functions rather independently from the DsbA-DsbB system and that unlike DsbA, the DsbC redox-active site is not recycled by the inner membrane DsbB protein (60). A *dsbC* null defect more severely impairs the folding of proteins with multiple disulfide bonds, such as AP and penicillin-binding protein 4, in which cases the requirement for an isomerase activity is greater (60). Similarly, it has quite recently been shown that the folding of mouse urokinase, which contains 12 disulfide bonds, is severely affected in a *dsbC* null mutant, again arguing the importance of DsbC isomerase activity in vivo (68). Although folding defects in a *dsbC* null mutant are not always as dramatic as those in a *dsbA* null mutant, they are severe enough to trigger the specific σ^E-dependent regulon, which is induced upon misfolding of periplasmic proteins (56, 57, 67).

Under normal growth conditions, oxidation of proteins does not appear to be a limiting factor in *E. coli*. The DsbA-DsbB system provides an excellent oxidative potential. For example, *dsbB* alone on a medium-copy plasmid allows bacteria to cope with lethal concentrations of DTT (61). However, an imbalance in the ratio between oxidized and reduced forms of both DsbA and DsbC, as observed in bacteria carrying mutations in the *dsbD* gene, leads to defects in proper disulfide bond formation of transported proteins, particularly those with multiple disulfide bonds, such as human AP (5) or BPTI (19). Since both DsbA and DsbC accumulate more in the oxidized state in a *dsbD* mutant (59), it is likely that excessive oxidation of disulfide-containing proteins is mediated by DsbA and DsbC in a random manner. These observations are in agreement with the presumed thiol:disulfide reductase function of DsbD. Based on the available genetic data (59) and preliminary biochemical evidence, it is quite likely that DsbC isomerase activity directly depends on DsbD. Indeed, such an activity implies that the thiol group of Cys^{98} in the active site of DsbC is free and accessible (Fig. 1). DsbD may be responsible for maintaining Cys^{98} in its thiolate form. Synergism between DsbC and DsbD is further supported by the fact that a *dsbD* null mutation suppresses only the phenotypic defects associated with a *dsbA* or *dsbB* mutant background and not those of *dsbC* mutants. Such *dsbD* null suppressors partly alleviate the lethal growth phenotype of *dsbA* mutants (but not of *dsbC* mutant bacteria) which is observed when these mutant cells are grown in the presence of DTT or benzylpenicillin (60). Consistent with a common DsbC- and DsbD-mediated pathway, some additivity is observed in *dsbC dsbD* double null mutant bacteria (59). Finally, the suppression effect of a *dsbD* null mutation in a *dsbA* mutant strain is dependent solely on the presence of a wild-type DsbC protein which accumulates in its oxidized form (59, 68).

Extragenic suppressor analyses of *dsbD* mutant bacteria have identified yet another *dsb* gene, designated *dsbE*. For example, the stress response induced upon accumulation of misfolded proteins in *dsbD* mutant bacteria (59) is suppressed by overexpressing DsbE. DsbE is a soluble periplasmic protein with a Cys-Pro-Thr-Cys active site (Fig. 1). As stated earlier, null mutations in *dsbE* were also independently isolated on the basis of suppression of the DTT-sensitive phenotype of *dsbA* null mutant bacteria. Biochemical data suggest that both DsbE and DsbD have redox properties closer to those of thioredoxin than either DsbA or DsbC (62). This explains how multiple copies of *dsbE* in a *dsbD* null mutant could correct the otherwise excessive oxidation processes mediated by DsbA and DsbC. The fact that a maturation defect of *c*-type cytochromes is also observed in *dsbE* mutant bacteria (62), as is the case with *dsbD* (*dipZ*) mutants (13), suggests that both proteins are also involved in common cellular processes.

A global model illustrating the proposed roles of the different Dsb proteins is shown in Fig. 3.

Role of the Dsb proteins in cytochrome biogenesis. The *c*-type cytochromes are electron transfer proteins found in the respiratory chains of both eukaryotes and prokaryotes. *E. coli* can synthesize at least five *c*-type cytochromes, depending on the availability of electron acceptors (34). It is interesting that some of the genes, like *helX* in *Rhodobacter capsulatus* (6) or *tlpA* in *Bradyrhizobium japonicum*, whose products are essential for the maturation of *c*-type cytochromes, belong to the thioredoxin superfamily (47). In *E. coli*, the *dsbD* and *dsbA* genes have also been shown to be essential for formate-dependent nitrite reductase (Nrf) activity and *c*-type cytochrome biogenesis (13, 54). These studies were based on independent searches for null mutations which either lack Nrf activity or exhibit defects in *c*-type cytochrome biogenesis. It is likely that DsbD maintains the cysteine residues of the apocytochrome in a reduced state to allow proper covalent linkage with the heme. It has recently been shown that DsbB is also required for the synthesis of *c*-type cytochromes (53). An important additional finding from these studies has been the observation that unlike *dsbA* mutants, *dsbB* mutant bacteria are also defective in periplasmic nitrate reductase activity, indicating an additional role for DsbB in anaerobic electron transport (53).

MISFOLDING OF EXPORTED PROTEINS: INDUCTION OF THE σ^E REGULON

In the cytoplasm of bacteria, protein misfolding triggers the classical σ^{32}-dependent heat shock response (8, 20). The transient increase in the level of the σ^{32} polypeptide allows a rapid increase in the transcription of heat shock genes. Some of these genes encode the major chaperones which are essential

for coping with the accumulation of heat-denatured proteins. It is clear that folding catalysts and specific chaperones are also present in the periplasmic space. It is less clear whether misfolding, aggregation, or heat denaturation of exported proteins also induces the σ^{32}-dependent heat shock response. In most cases, it may not. For example, altering the solubility of two periplasmic proteins, L-asparaginase II (AnsB) and the maltose carrier protein (MalE), by synthesizing a truncated AnsB* protein or a mutant variant of MalE* which forms inclusion bodies in the periplasm, does not induce the transcription of either *lon* or *groE*, two genes transcribed by the $E\sigma^{32}$ polymerase (57).

The initial hint regarding a possible response to the accumulation of misfolded protein in the cell envelope, came from various observations. Several pieces of biochemical evidence demonstrated the existence of an additional sigma factor in *E. coli*, designated either σ^E or σ^{24}. This factor was found to ensure the sustained transcription of the *rpoHP3* promoter at elevated temperatures (*rpoH* encodes the σ^{32} factor [16, 79]). Initial attempts to isolate the *rpoE* gene were based on the assumption that multicopy expression of *rpoE* should lead to increased β-gal activity from an *rpoHP3-lacZ* transcriptional fusion. Quite unexpectedly, this multicopy approach revealed that overexpression of some OMPs and the consequent imbalance in the ratio of OMPs are inducers of the σ^E regulon (52, 69).

These initial findings were further sustained with the cloning of the *rpoE* gene encoding σ^E (67, 69). Interestingly, the sequence of the σ^E polypeptide showed extended homology to a subfamily of sigma factors, designated extracytoplasmic factors, which are devoted to the regulation of extracytoplasmic activities (48). Closer examination of the stimuli leading to the σ^E-dependent response in *E. coli* revealed that misfolding or alteration of most transported proteins, whether destined for the outer membrane or the extracellular medium or simply residing in the periplasm, induces the σ^E regulon (56, 57, 67). Hence, the periplasmic truncated AnsB* protein or the aggregation-prone MalE* protein exclusively induces transcription from the *rpoHP3*, *rpoEP2*, or *htrA* promoters, all of which are recognized by the $E\sigma^E$ polymerase (57). Similarly, mutations in any of the *dsb* genes also trigger a σ^E-dependent response, presumably because they lead to the accumulation of slow-folding species in the periplasm. It is thus pertinent to point out that mutations in any of the *dsb* genes can be isolated by simply scoring for increased σ^E activity. What is even more important is that the induction of the σ^E-dependent response is even more dramatic if a null mutation in any of the *dsb* genes is combined with an *htrA* null mutation (57, 67). In fact, *htrA* encodes a periplasmic protease (HtrA-DegP) which has been shown to degrade abnormal polypeptides (45, 74, 75). The molecular mechanism of signal transduction pathways in response to protein misfolding in *E. coli*'s periplasm has been recently reviewed in more detail (55).

THE OTHER FOLDING CATALYSTS IN THE PERIPLASM

Besides the Dsb proteins, the periplasm contains other catalysts of folding. At least one PPI, RotA (46), has been identified in the periplasm, although its role in protein folding in vivo is still not known. No noticeable growth or folding defect has been observed in *rotA* mutant bacteria (42). As discussed above, accumulation of unfolded proteins in the periplasm or an altered membrane composition leads to constitutive elevated expression of the σ^E regulon. Such an induction is not observed in *rotA* mutant bacteria (57).

Pilus subunits (Pap), while transiting through the periplasm, require the presence of two specific extracytoplasmic proteins, PapC and PapD, respectively designated molecular usher and chaperone proteins (32). PapC and PapD prevent degradation of the various pilus subunits before their assembly at the outer membrane.

A global search for mutations that lead to elevated expression of the σ^E regulon identified again the various *dsb* genes, as well as new folding catalysts (57). This search was combined with a second approach which involved screening of multicopy libraries for genes that lead to a decrease in the σ^E-dependent response induced by misfolded proteins or by mutants with leaky membranes (e.g., *htrM* mutants which produce a defective lipopolysaccharide [LPS]). These two approaches identified three genes, *surA*, *fkpA*, and *skp/ompH*, whose products play an active role either as folding catalysts or as chaperones in the periplasm (57). SurA and FkpA have been purified and, consistent with their predicted amino acid sequences, have been shown to be bona fide PPIs (57). SurA is highly similar to the parvulin family of PPIs and contains two repeats of a parvulin-like domain at its C-terminal end (70). In contrast, FkpA resembles the classical FK506 binding proteins, a class of highly conserved PPIs. *surA* had been identified earlier as a gene whose product is required for survival of *E. coli* during the stationary phase (77). A chaperone cum folding catalyst role for SurA has been proposed based on the multiple suppression effects observed with multicopy SurA (57). First, SurA is able to assist the folding of OMPs immediately after export, even in the absence of proper LPS. Second, SurA in multicopy can promote the folding of many otherwise unstable proteins, such as a protein A–β-lactamase hybrid protein or aggregation-prone proteins like mutant MalE31 and truncated periplasmic AnsB*. Finally, SurA can restore to nearly the wild-type level the σ^E-dependent response otherwise constitutively induced by misfolded proteins accumulating in bacteria simultaneously carrying mutations in *htrA* and one of the *dsb* genes. Also, *surA* mutant bacteria present major defects in the maturation of OMPs and, as a consequence, exhibit sensitivity to detergents (57). In fact, such mutants are unable to achieve complete folding of the OmpA, OmpF, and LamB proteins as determined by trypsin sensitivity (44).

A prominent role for Skp/OmpH during OMP folding is based on similar phenotypic defects observed in *surA* mutant bacteria in terms of the elevated σ^E-dependent response, as well as defects in membranes (57). It has been recently shown that Skp can bind selectively to certain OMPs, suggesting its chaperone-like role (10). Given the known highly basic nature of this protein and previous copurification of Skp/OmpH with LPS (22), it has been proposed that Skp may act as an exchange factor, which helps either to remove or to exchange the original LPS molecule associated with either the monomers or protrimers of OMPs (57; Fig. 4). In vivo, this exchange of LPS may trigger the insertion of OMPs into the outer membranes and thereby accelerate their final folded conformation as integrated membrane proteins.

CONCLUSIONS

It seems clear that protein folding is an assisted process, whether it is in the cytoplasm or in the periplasm. Considering the need to maintain a relatively more oxidizing environment in the periplasm, we have seen that *E. coli* and other gram-negative bacteria synthesize redox proteins like DsbA and DsbB, which act as strong thiol:disulfide oxidants. What has emerged, quite surprisingly, is that the periplasm also contains thiol:disulfide reductases like DsbD and DsbE, which are

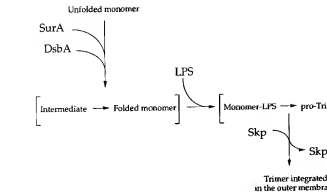


FIG. 4. Proposed model for the folding of OMPs.

needed both to maintain a proper redox state for true periplasmic disulfide isomerases like DsbC and for c-type cytochrome maturation. In addition, the periplasm also contains at least three PPIs, RotA, FkpA, and SurA. Of these, SurA, along with another periplasmic protein, Skp, may have, in addition, chaperone-like properties that are essential for outer membrane biogenesis.

REFERENCES

1. **Alksne, L. E., D. Keeney, and B. A. Rasmussen.** 1995. A mutation in either *dsbA* or *dsbB*, a gene encoding a component of a periplasmic disulfide bond-catalyzing system, is required for high-level expression of the *Bacteroides fragilis* metallo-β-lactamase, CcrA, in *Escherichia coli*. J. Bacteriol. 177:462–464.
2. **Åslund, F., B. Ehn, A. Miranda-Vizuet, C. Pueyo, and A. Holmgren.** 1994. Two additional glutaredoxins exist in Escherichia coli: glutaredoxin 3 is a hydrogen donor for ribonucleotide reductase in a thioredoxin/glutaredoxin 1 double mutant. Proc. Natl. Acad. Sci. USA 91:9813–9817.
3. **Bardwell, J. C. A., J.-O. Lee, G. Jander, N. Martin, D. Belin, and J. Beckwith.** 1993. A pathway for disulfide bond formation *in vivo*. Proc. Natl. Acad. Sci. USA 90:1038–1042.
4. **Bardwell, J. C. A., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation *in vivo*. Cell 67:581–589.
5. **Beck, B., H. Crooke, M. Jarsch, J. Cole, and H. Burtscher.** 1994. Mutation in *dipZ* leads to reduced production of active human placental alkaline phosphatase in *Escherichia coli*. FEMS Microbiol. Lett. 124:209–214.
6. **Beckman, D. L., and R. G. Kranz.** 1993. Cytochrome c biogenesis in a photosynthetic bacterium requires a periplasmic thioredoxin-like protein. Proc. Natl. Acad. Sci. USA 90:2179–2183.
7. **Belin, P., and P. L. Boquet.** 1993. A second gene involved in the formation of disulfide bonds in proteins localized in *Escherichia coli* periplasmic space. C. R. Acad. Sci. III 316:469–473.
8. **Bukau, B.** 1993. Regulation of the *Escherichia coli* heat-shock response. Mol. Microbiol. 9:671–680.
9. **Bushweller, J. H., F. Åslund, K. Wüthrich, and A. Holmgren.** 1992. Structural and functional characterization of the mutant *Escherichia coli* glutaredoxine (C14-S) and its mixed disulfide with glutathione. Biochemistry 31:9288–9293.
10. **Chen, R., and U. Henning.** 1996. A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. Mol. Microbiol. 19:1287–1294.
11. **Creighton, T. E.** 1986. Detection of folding intermediates using urea-gradient electrophoresis. Methods Enzymol. 131:156–172.
12. **Creighton, T. E., and J. Goldenberg.** 1984. Kinetic role of a meta-stable native-like two-disulphide species in the folding transition of bovine pancreatic trypsin inhibitor. J. Mol. Biol. 179:497–526.
13. **Crooke, H., and J. Cole.** 1995. The biogenesis of c-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. Mol. Microbiol. 15:1139–1150.
14. **Dailey, F. E., and H. C. Berg.** 1993. Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 90:1043–1047.
15. **Ellis, L. B., P. Saurugger, and C. Woodward.** 1992. Identification of the three-dimensional thioredoxin motif: related structure in the ORF3 protein of the *Staphylococcus aureus mer* operon. Biochemistry 31:4882–4891.
16. **Erickson, J. W., and C. A. Gross.** 1989. Identification of the σ^E subunit of *Escherichia coli* RNA polymerase; a second alternate σ factor involved in high temperature gene expression. Genes Dev. 3:1462–1471.
17. **Fong, S.-T., J. Camakaris, and B. T. O. Lee.** 1995. Molecular genetics of a chromosomal locus involved in copper tolerance in *Escherichia coli* K-12. Mol. Microbiol. 15:1127–1137.
18. **Freedman, R. B.** 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell 57:1069–1072.
19. **Georgiou, G.** Personal communication.
20. **Georgopoulos, C.** 1992. The emergence of the chaperone machines. Trends Biochem. Sci. 17:295–299.
21. **Gething, M.-J., and J. Sambrook.** 1992. Protein folding in the cell. Nature 355:33–45.
22. **Geyer, R., C. Galanos, O. Westphal, and J. R. Golecki.** 1979. A lipopolysaccharide-binding cell-surface protein from *Salmonella minnesota*. Isolation, partial characterization and occurrence in different Enterobacteriaceae. Eur. J. Biochem. 98:27–38.
23. **Glockshuber, R.** Personal communication.
24. **Goldberger, R. F., C. J. Epstein, and C. B. Anfinsen.** 1963. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. 238:628–635.
25. **Grauschopf, U., J. R. Winther, P. Korber, T. Zander, P. Dallinger, and J. C. Bardwell.** 1995. Why is DsbA such an oxidizing disulfide catalyst? Cell 83:947–955.
26. **Gross, C. A.** 1996. Function and regulation of the heat shock proteins, p. 1382–1399. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schalchter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, American Society for Microbiology, Washington, D.C.
27. **Guilhot, C., G. Jander, N. L. Martin, and J. Beckwith.** 1995. Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA. Proc. Natl. Acad. Sci. USA 92:9895–9899.
28. **Hawkins, H. C., M. DeNardi, and R. B. Freedman.** 1991. Redox properties and cross-linking of the dithiol/disulfide active sites of mammalian protein disulfide isomerase. Biochem. J. 275:349–353.
29. **Hendrick, J. P., and F. U. Hartl.** 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. 62:349–384.
30. **Holmgren, A.** 1989. Thioredoxin and glutaredoxin systems. J. Biol. Chem. 264:13963–13966.
31. **Holmgren, A.** 1985. Thioredoxin. Annu. Rev. Biochem. 54:237–271.
32. **Hultgren, S. J., F. Jacob-Dubuisson, C. H. Jones, and C. I. Branden.** 1993. PapD and superfamily of periplasmic immunoglobulin-like pilus chaperones. Adv. Protein Chem. 44:99–123.
33. **Hwang, C., A. J. Sinskey, and H. F. Lodish.** 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. Science 257:1496–1502.
34. **Iobbi-Nivol, C., H. Crooke, L. Griffiths, J. Grove, H. Hussain, J. Pommier, V. Méjean, and J. A. Cole.** 1994. A re-assessment of the range of c-type cytochromes synthesized by *Escherichia coli* K-12. FEMS Microbiol. Lett. 119:89–94.
35. **Ishihara, T., H. Tomita, Y. Hasegawa, N. Tsukagoshi, H. Yamagata, and S. Udaka.** 1995. Cloning and characterization of the gene for a protein thiol-disulfide oxidoreductase in *Bacillus brevis*. J. Bacteriol. 177:745–749.
36. **Jander, G., N. L. Martin, and J. Beckwith.** 1994. Two cysteines in each periplasmic domain of the membrane protein DsbB are required for its function in protein disulfide bond formation. EMBO J. 13:5121–5127.
37. **Kamitani, S., Y. Akiyama, and K. Ito.** 1992. Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. EMBO J. 11:57–62.
38. **Kelley, R. F., W. Shalongo, M. V. Jagannadham, and E. Stellwagen.** 1987. Equilibrium and kinetic measurements of the conformational transition of reduced thioredoxin. Biochemistry 26:1406–1411.
39. **Kemmink, J., N. J. Darby, K. Dijkstra, R. Mark, M. Scheek, and T. E. Creighton.** 1995. Nuclear magnetic resonance characterization of the N-terminal thioredoxin-like domain of protein disulfide isomerase. Protein Sci. 4:2587–2593.
40. **Kishigami, S., and K. Ito.** 1996. Roles of cysteine residues of DsbB in its activity to reoxidize DsbA, the protein disulphide bond catalyst of *Escherichia coli*. Genes Cells 1:201–208.
41. **Kishigami, S., E. Kanaya, M. Kikuchi, and K. Ito.** 1995. DsbA-DsbB interaction through their active site cysteines. J. Biol. Chem. 270:17072–17074.
42. **Kleerebezem, M., M. Heutink, and J. Tommassen.** 1995. Characterization of an *Escherichia coli rotA* mutant, affected in periplasmic peptidyl-prolyl cis/trans isomerase. Mol. Microbiol. 18:313–320.
43. **Krause, G., J. Lundström, J. L. Barea, C. de la Cuesta, and A. Holmgren.** 1991. Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in *Escherichia coli* thioredoxin. J. Biol. Chem. 266:9494–9500.
44. **Lazar, S., and R. Kolter.** 1996. SurA assists the folding of *Escherichia coli* outer membrane proteins. J. Bacteriol. 178:1770–1773.
45. **Lipinska, B., S. Sharma, and C. Georgopoulos.** 1988. Sequence analysis and regulation of the *htrA* gene of *Escherichia coli*: a sigma 32-independent mechanism of heat-inducible transcription. Nucleic Acids Res. 16:10053–10067.
46. **Liu, J., and C. T. Walsh.** 1990. Peptidyl-prolyl cis-trans-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. Proc. Natl. Acad. Sci. USA 87:4028–4032.

47. **Loferer, H., M. Bott, and H. Hennecke.** 1993. *Bradyrhizobium japonicum* TlpA, a novel membrane-anchored thioredoxin-like protein involved in the biogenesis of cytochrome aa3 and development of symbiosis. EMBO J. 12:3373–3383.
48. **Lonetto, M., K. L. Brown, K. E. Rudd, and M. J. Buttner.** 1994. Analysis of the *Streptomyces coelicolor* sigmaE gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA **91**:7573–7577.
49. **Lundström, J., G. Krause, and A. Holmgren.** 1992. A Pro to His mutation in active site of thioredoxin increases its disulfide-isomerase activity 10-fold. New refolding systems for reduced or randomly oxidized ribonuclease. J. Biol. Chem. 267:9047–9052.
50. **Martin, J. L., J. C. A. Bardwell, and J. Kuriyan.** 1993. Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*. Nature 365: 464–468.
51. **Mayhew, M., A. C. da Silva, J. Martin, H. Erdjument-Bromage, P. Tempst, and F.-U. Hartl.** 1996. Protein folding in the central cavity of the GroEL-GroES chaperonin complex. Nature 379:420–426.
52. **Mecsas, J., P. E. Rouvière, J. W. Erickson, T. J. Donohue, and C. A. Gross.** 1993. The activity of σ^E, an *Escherichia coli* heat-inducible sigma factor, is modulated by expression of outer membrane proteins. Genes Dev. 7:2618–2628.
53. **Metheringham, R., K. Tyson, H. Crooke, D. Missiakas, S. Raina, and J. Cole.** 1996. Effects of mutations in genes for proteins involved in disulfide bond formation in the periplasm on the activities of anaerobically induced electron transfer chains in *Escherichia coli* K-12. Mol. Gen. Genet. **253**:95–102.
54. **Metheringham, R., L. Griffiths, H. Crooke, S. Forsythe, and J. Cole.** 1995. An essential role for DsbA in cytochrome c synthesis and formate-dependent nitrite reduction by *Escherichia coli* K-12. Arch. Microbiol. 164:301–307.
55. **Missiakas, D., and S. Raina.** 1997. Signal transduction pathways in response to protein misfolding in *E. coli*'s cell envelope. Trends Biochem. Sci. **21**:59–63.
56. **Missiakas, D., S. Raina, and C. Georgopoulos.** 1996. The heat shock regulation, p. 481–501. *In* E. C. C. Lin and S. A. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Co., Austin, Tex.
57. **Missiakas, D., J.-M. Betton, and S. Raina.** 1996. New components of protein folding in the extra-cytoplasmic compartments of *Escherchia coli*: SurA, FkpA and Skp/OmpH. Mol. Microbiol. 21:871–884.
58. **Missiakas, D., and S. Raina.** DsbE. *In* M.-J. Gething and J. Samhrook (ed.), Guidebook to molecular chaperones and protein folding catalysts, in press. Oxford University Press, Oxford, England.
59. **Missiakas, D., F. Schwager, and S. Raina.** 1995. Identification and characterization of a new disulfide-isomerase like protein (DsbD) in *Escherichia coli*. EMBO J. 14:3415–3424.
60. **Missiakas, D., C. Georgopoulos, and S. Raina.** 1994. The *Escherichia coli dsbC* (*xprA*) gene encodes a periplasmic protein involved in disulfide bond formation. EMBO J. 13:2013–2020.
61. **Missiakas, D., C. Georgopoulos, and S. Raina.** 1993. Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds *in vivo*. Proc. Natl. Acad. Sci. USA 90:7084–7088.
62. **Missiakas, D., W. English, T. E. Creighton, and S. Raina.** Unpublished data.
63. **Nelson, J. W., and T. E. Creighton.** 1994. Reactivity and ionization of the active site cysteine residues of DsbA, a protein required for disulfide bond formation *in vivo*. Biochemistry 33:5974–5983.
64. **Okamoto, K., T. Baba, H. Yamanaka, N. Akashi, and Y. Fujii.** 1995. Disulfide bond formation and secretion of *Escherichia coli* heat-stable enterotoxin II. J. Bacteriol. 177:4579–4586.
65. **Ostermeier, M., K. De Sutter, and G. Georgiou.** 1996. Eukaryotic protein disulfide isomerase complements *Escherichia coli dsbA* mutants and increases the yield of a heterologous secreted protein with disulfide bonds. J. Biol. Chem. 271:10616–10622.
66. **Peek, J. A., and R. K. Taylor.** 1992. Characterization of a periplasmic thiol: disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **89**: 6210–6214.
67. **Raina, S., D. Missiakas, and C. Georgopoulos.** 1995. The *rpoE* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*. EMBO J. 14:1043–1055.
68. **Rietsch, A., D. Belin, N. Martin, and J. Beckwith.** 1996. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 93:13048–13053.
69. **Rouvière, P., A. de las Penas, J. Mecsas, C. Z. Lu, K. E. Rudd, and C. A. Gross.** 1995. *rpoE*, the gene encoding the second heat-shock sigma factor, σ^E, in *Escherichia coli*. EMBO J. 14:1032–1042.
70. **Rudd, K. E., H. J. Sofia, E. V. Koonin, G. Plunkett, S. Lazar, and P. E. Rouvière.** 1995. New family of peptidyl-prolyl isomerases. Trends Biochem. Sci. 20:12–14.
71. **Russel, M.** 1995. Thioredoxin genetics. Methods Enzymol. 252:264–274.
72. **Rybin, V., A. Zapun, A. Törrönen, S. Raina, D. Missiakas, T. E. Creighton, and P. Metcalf.** 1996. Crystallization of DsbC, the disulphide bond isomerase of *Escherichia coli*. Acta Crystallogr. Sect. D Biol. Crystallogr. 52:1219–1221.
73. **Shevchik, V. E., G. Condemine, and J. Robert-Baudouy.** 1994. Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. EMBO J. 13:2007–2012.
74. **Strauch, K. L., and J. Beckwith.** 1988. An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. USA 85:1576–1580.
75. **Strauch, K. L., K. Johnson, and J. J. Beckwith.** 1989. Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. J. Bacteriol. 171:2689–2696.
76. **Tomb, J.-F.** 1992. A periplasmic protein disulfide oxidoreductase is required for transformation of *Haemophilus influenzae* Rd. Proc. Natl. Acad. Sci. USA 89:10252–10256.
77. **Tormo, A., M. Almiron, and R. Kolter.** 1990. *surA*, an *Escherichia coli* gene essential for survival in stationary phase. J. Bacteriol. 172:4339–4347.
78. **Venetianer, P., and F. B. Straub.** 1963. The enzymic reactivation of reduced ribonuclease. Biochim. Biophys. Acta 67:166–168.
79. **Wang, Q., and J. M. Kaguni.** 1989. A novel sigma factor is involved in expression of the *rpoH* gene of *Escherichia coli*. J. Bacteriol. 171:4248–4253.
80. **Weissman, J. S., H. S. Rye, W. A. Fenton, J. M. Beechem, and A. L. Horwich.** 1996. Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. Cell 84:481–490.
81. **Wunderlich, M., and R. Glockshuber.** 1993. Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. Protein Sci. 2:717–726.
82. **Wunderlich, M., A. Otto, R. Seckler, and R. Glockshuber.** 1993. Bacterial protein disulfide isomerase: efficient catalysis of oxidative protein folding at acidic pH. Biochemistry 32:12251–12256.
83. **Yamanaka, H., M. Kameyama, T. Baba, Y. Fujii, and K. Okamoto.** 1994. Maturation pathway of *Escherichia coli* heat-stable enterotoxin I: requirement of DsbA for disulfide bond formation. J. Bacteriol. 176:2906–2913.
84. **Yu, J., S. McLaughlin, R. B. Freedman, and T. R. Hirst.** 1993. Cloning and active site mutagenesis of *Vibrio cholerae* DsbA, a periplasmic enzyme that catalyzes disulfide bond formation. J. Biol. Chem. 268:4326–4330.
85. **Yu, J., H. Webb, and T. R. Hirst.** 1992. A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in *Vibrio cholerae*. Mol. Microbiol. 6:1949–1958.
86. **Zapun, A., D. Missiakas, S. Raina, and T. E. Creighton.** 1995. Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. Biochemistry 34:5075–5089.
87. **Zapun, A., L. Cooper, and T. E. Creighton.** 1994. Replacement of the active-site cysteine residues of DsbA, a protein required for disulfide bond formation *in vivo*. Biochemistry 33:1907–1914.
88. **Zapun, A., J. C. A. Bardwell, and T. E. Creighton.** 1993. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo*. Biochemistry 32:5083–5092.